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PR-394

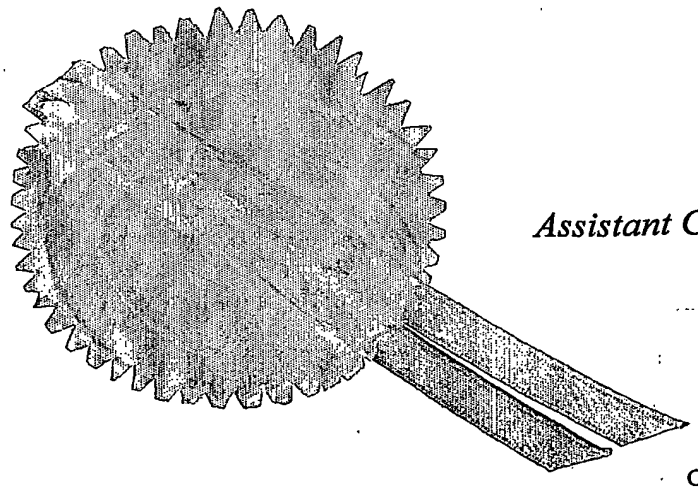
सत्यमेव जयते

INTELLECTUAL PROPERTY INDIA

GOVERNMENT OF INDIA
MINISTRY OF COMMERCE & INDUSTRY,
PATENT OFFICE, DELHI BRANCH,
W - 5, WEST PATEL NAGAR,
NEW DELHI - 110 008.

I, the undersigned being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application, Complete Specification*** *and* ***Drawing Sheets*** *filed in connection with Application for Patent No.* ***559/Del/03*** *dated* ***01st April 2003.***

Witness my hand this 17th day of May 2004.



(S.K. PANGASA)
Assistant Controller of Patents & Designs

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Chemistry
C12N15/53

0559-03

- 1 APR 2003

FORM 1

THE PATENTS ACT, 1970

(39 of 1970)

APPLICATION FOR GRANT OF A PATENT

(See Sections 7, 54 and 135 and rule 33A)

1 We, **RANBAXY LABORATORIES LIMITED**, a Company incorporated under the Companies Act, 1956 of 19, Nehru Place, New Delhi - 110 019, India

2. hereby declare –

(a) that we are in possession of an invention titled **"AN IMPROVED FERMENTATION PROCESS FOR THE PREPARATION OF PRAVASTATIN"**

(b) that the Complete Specification relating to this invention is filed with this application.

(c) that there is no lawful ground of objection to the grant of a patent to us.

3. Further declare that the inventors for the said invention are

a. PARVEEN KUMAR
b. VIKAS KATIAL
c. PARESH GIGRAS
d. SUDEEP KUMAR
e. ANIRUDDHA SHUKLA

of Ranbaxy Laboratories Limited, Plot No. 20, Sector-18, Udyog Vihar Industrial Area, Gurgaon – 122001 (Haryana), India, all Indian Nationals.

4. That we are the assignee or legal representatives of the true and first inventors.

5. That our address for service in India is as follows:

DR. B. VIJAYARAGHAVAN
Group Leader – Intellectual Property
Ranbaxy Laboratories Limited
Plot No.20, Sector – 18,
Udyog Vihar Industrial Area,
Gurgaon – 122001 (Haryana).
INDIA.
Tel. No. (91-124) 2343126, 2342001 – 10; 5012501-10
Fax No. (91-124) 2342027 ; 2343545

6. Following declaration was given by the inventors in the convention country:

We, PARVEEN KUMAR, VIKAS KATIAL, PARESH GIGRAS, SUDEEP KUMAR, ANIRUDDHA SHUKLA of Ranbaxy Laboratories Limited, Plot No. 20, Sector – 18, Udyog Vihar Industrial Area, Gurgaon–122001 (Haryana), India, all Indian Nationals, the true and first inventors for this invention in the convention country declare that the applicants herein, **Ranbaxy Laboratories Limited**, 19, Nehru Place, New Delhi - 110 019, India, is our assignee or legal representatives.

a. (PARVEEN KUMAR)

b. (VIKAS KATIAL)

c. (PARESH GIGRAS)

d. (SUDEEP KUMAR)

e. (ANIRUDDHA SHUKLA)

7. That to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

8. Followings are the attachment with the application:

a. Complete Specification (3 copies)
b. Drawings (3 copies)
c. Statement and Undertaking on FORM – 3
d. Fee Rs.5,000/- (Rupees Five Thousand only..) in cheque bearing No. **688613** dated :**17.03.2003** on **ANZ Grindlays Bank, New Delhi.**

We request that a patent may be granted to us for the said invention.

Dated this **1ST** day of **April, 2003.**

For Ranbaxy Laboratories Limited

(SUSHIL KUMAR PATAWARI)
COMPANY SECRETARY

FORM 2

The Patents Act, 1970

(39 of 1970)

COMPLETE SPECIFICATION
(See Section 10)

AN IMPROVED FERMENTATION PROCESS FOR THE PREPARATION OF PRAVASTATIN

RANBAXY LABORATORIES LIMITED
19, NEHRU PLACE, NEW DELHI-110019

A Company incorporated under the Companies Act, 1956

The following specification particularly describes and ascertains the nature of this invention and the manner in which it is to be performed:

The present invention provides an improved fermentation process for the preparation of pravastatin. The process describes a method of producing pravastatin by microbial hydroxylation of compactin (ML-236B) by substantially reducing the related impurities formed during the process, by maintaining a residual level of compactin.

Hypercholesterolemia or elevated plasma cholesterol level has long been recognized as a major risk factor for atherosclerotic disease, and specifically for coronary heart disease. Biosynthesis of cholesterol is a major contributing factor to hypercholesterolemia. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate in the rate determining step in the biosynthesis of cholesterol. It was expected that plasma cholesterol could be reduced as a result of inhibition of HMG-CoA reductase because more than 70% of the total input of body cholesterol is derived from de novo synthesis in humans.

Pravastatin, simvastatin, lovastatin, mevastatin, atorvastatin, fluvastatin, cerivastatin and derivatives and analogues thereof are known as HMG-CoA reductase inhibitors and are used as antihypercholesterolemic agents.

During the past two decades, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) has been extensively studied. In 1975, compactin (ML-236B), a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in the

biosynthesis of cholesterol, was discovered in the culture broth of *Pencillium citrinum.* After thorough screening of hundreds of microbial products as well as chemically or biologically modified derivatives of ML-236B, pravastatin sodium was chosen as a candidate for development because of its stronger and more tissue-selective activity than the prototype compound.

Presently, the most economically feasible process for making pravastatin is by the microbial hydroxylation of compactin at C-6 position.

Microbial hydroxylation of compactin to pravastatin can be accomplished to various extents with molds belonging to different genera viz *Mucor Rhizopus, Syncephalastrum, Cunninghamella, Martierella* and with filamentous bacteria belonging to different genera viz *Nocardia, Actinomadura, Streptomyces*, as described in various patents [US patents 5,179,013; 4,448,979; 4,346,227; 4,537,859; US pub no. US2002/0081675A1; US2001/0026934A1; Japanese patent 58-10572; European patent 0605230].

The most common problem encountered in the commercial production of pravastatin is that most microorganisms are not able to tolerate the compactin substrate fed even at low concentrations due to its cytotoxic effect. (Biotechnol. Bioeng., 42:815-820, 1993]. Taking into account the efficient manufacture of the active ingredient on an industrial scale, it is

important to have a strain that is able to tolerate high concentrations of compactin as well as pravastatin.

Furthermore, the purity of the active ingredient is an important factor for manufacturing a safe and effective pharmaceutical product. Several structurally related products are sometimes produced in fermentation processes, and often only one product is desired from a process, thereby requiring a method to control the ratio of these different analogues. A commercial process typically requires either exclusive or predominant production of one product. While the nature of polyketide synthase permits controlled biosynthesis of single chemical entity, a significant number of polyketide synthases generate related products (J. Ind. Microbiol. Biotechnol., 27; 368-377, 2001). However, the known methods of producing pravastatin are ill-suited in controlling the formation of these analogues.

The present invention meets the need for an improved microbial process for preparing pravastatin by substantially reducing the dihydroxy impurities and analogues thereof, formed during the process.

The aim of the present invention is to provide a new and efficient method of microbial hydroxylation of compactin on an industrial scale at more advantageous conditions than those previously known to the one skilled in the art. During the research work, the inventors worked on a microbial strain with a hydroxylase enzyme system that was well adapted for the

microbial transformation of compactin to pravastatin at high concentrations. The present invention relates to a microbial process for the preparation of pravastatin substantially reducing the dihydroxy impurities and analogues thereof, formed during the bioconversion process, by maintaining a minimal residual level of compactin.

In the present invention, the residual compactin level is maintained, resulting in ~ 14 fold decrease in 3"-hydroxy pravastatin (Impurity B as per European Pharmacopeia; Formula-III) and ~ 7 fold decrease in 3"S hydroxy pravastatin (Formula-IV).

Accordingly, the present invention provides an efficient method of producing substantially pure pravastatin of structural formula I by microbial hydroxylation of compactin of structural formula II and substantially reducing the quantities of the derivatives thereof as well as analogues and structurally related compounds, namely 3"hydroxy pravastatin and its isomer of structural formula III and IV respectively formed during the process, by maintaining the residual level of compactin during the process.

The present invention provides a method for converting compactin to pravastatin. Compactin is provided and contacted with whole cells of *Streptomyces* sp. under the conditions in which the agent converts compactin to pravastatin.

The method of present invention may be carried out subsequent to the growth of the microorganism employed and contacting with compactin as substrate, which enables the agent to convert compactin to pravastatin. Compactin is provided in the form of solution comprising sodium salt of compactin, which may be the purified / crude or intermediate stage of compactin.

The conversion reaction of compactin to pravastatin can be done using conditions, which will result in the production of pravastatin eg. fermentation technique most preferably of the type useful for large scale industrial fermentation process eg. Batch, fed batch or continuous culture. Preferably an agitated liquid submerged culture is used.

The growth of the microorganism may be achieved by use of an appropriate medium containing nutrients such as carbon, nitrogen sources and trace elements. Exemplary assimilable carbon sources include glucose, glycerol, maltose, dextrin, starch, sucrose etc. and nitrogen sources include soybean meal, peptones, cotton seed meal, corn steep liquor, meat extract, yeast extract, ammonium sulfate, ammonium nitrate etc and inorganic salts as sodium chloride, phosphates, calcium carbonate etc are added in culture medium.

In the present invention the composition of the seed medium comprises of (g/L) glucose 16-25, soya bean meal 4-6, peptone 4-6, potassium phosphate 0.08-0.13 and calcium carbonate 4-6. Preferably, the seed

medium used is glucose 20, soya bean meal 5, peptone 5, potassium phosphate 0.1 and calcium carbonate 5.

The production medium in the present invention comprises of glucose 15-23, soya bean meal 25-38, cottonseed meal 2-4, corn steep liquor 5-8, sodium chloride 5-6 and calcium carbonate 2-3.

Preferably, the production medium used is (g/L) glucose 18, soya bean meal 30, cottonseed meal 3, corn steep liquor 6, sodium chloride 6 and calcium carbonate 2.4. The said preferred media for the growth of microorganism are those described in examples.

The preferred temperature is about 18°C to about 50°C, more preferably about 25°C to about 30°C and most preferably about 26°C to about 28°C. The preferred pH is about 5 to 10, more preferably about 6.0 to 8.5, most preferably about 7.3 to about 8.0. Preferably aerobic process e.g. by means of aeration and/or agitation. The preferred shaking condition is about 100 to 600 rpm more preferably about 100 to about 350 rpm and most preferably about 150 to 300 rpm.

The amount of starting material, compactin in the present process is preferably ranging from about 100 μg/mL to 1800 μg/mL of the culture medium, most preferably in the range of 300 μg/mL to 900 μg/mL.

The invention is meant to cover any percentage of conversion of compactin to pravastatin by *Streptomyces* sp. preferably at least about 50%, more preferably at least about 60%, most preferably about 65-75%. The following examples will illustrate the invention.

It should be noted that the media described in the Examples are merely illustrative of the wide variety of media, which may be employed, and yet are not intended to be limitative.

EXAMPLE –1

Bioconversion of Compactin to Pravastatin by Streptomyces sp.

This example illustrates the bioconversion of compactin to pravastatin by *Streptomyces carbophilus*. The seed medium was inoculated with slant culture and inoculated at 28°C for 2 days. 400 ml of mature seed culture was added to 20 L production fermenter.

The components of the seed and production medium employed in these examples are as follows:

A seed medium containing (g/L) glucose 20, soya bean meal 5, peptone 5, potassium phosphate 0.1 and calcium carbonate 5 was inoculated with spores of *Streptomyces* sp. from a slant culture, and cultured at 500 rpm, 28°C for 2 days, to give a seed culture. 400 ml of this seed culture was added to 20-L production fermenter containing (g/L) glucose 18,

soya bean meal 30, cottonseed meal 3, corn steep liquor 6, sodium chloride 6 and calcium carbonate 2.4.

The same or different media may be employed for different stages of growth. The medium was presterilized at 121°C for 30 minutes. After the growth of the microorganism, the initial shot of compactin was added to the fermenter to a level of 500 µg/ml and further cultured. Compactin was further added when the residual compactin level was ≤50 µg/ml. The rate of compactin bioconversion was monitored by HPLC. The % conversion was calculated on the basis of compactin added and pravastatin produced. The structurally related compounds, Impurity B (Formula III) and 3"S hydroxy pravastatin (Formula-IV) were also determined by HPLC.

The Impurity B (Formula III) and 3"S hydroxy pravastatin (Formula-IV) impurities obtained were in the range of 0.9 to 1.27 % (Fig.1) and 1.84 to 2.96 % (Fig. 2) respectively.

EXAMPLE –2

Bioconversion of Compactin to Pravastatin by *Streptomyces* sp. when residual compactin levels were maintained

The procedure of Example -1 was followed but the residual compactin level of 300-900 µg/ml was maintained through the batch as determined

by HPLC. Compactin was added when the level of residual compactin reached the lowest value in the range.

The Impurity B (Formula III) and 3"S hydroxy pravastatin (Formula-IV) impurities obtained were about 14 fold and 7 fold lower respectively than those mentioned in Example 1 (Fig 1 & 2).

The resultant purified product of example 2 using industrial scale recovery process (WO 01/44144 A2) shows better quality and limits the impurities to values which is better than the one reported in EP for active pharmaceutical composition as well as from the innovator.

EXAMPLE -3

Kinetics of bioconversion of compactin to pravastatin by *Streptomyces* sp.

This example illustrates the kinetics of conversion of Compactin to Pravastatin. The medium employed and culture conditions were the same as employed in Example 1. After 2 days of growth of microorganism, presterilized compactin solution was added to the fermenter to a level of 300-900 µg/ml and further cultured. Compactin was further added so as to maintain the compactin level in the prescribed range. The rate of compactin bioconversion at different time intervals was monitored for the next 6 days by HPLC. Percentage conversion was calculated on the basis of compactin charged and

pravastatin produced. The structurally related compounds, Impurity B (Formula-III) [Fig 1] and 3"S hydroxy pravastatin (Formula-IV) [Fig 2] were also determined by HPLC.

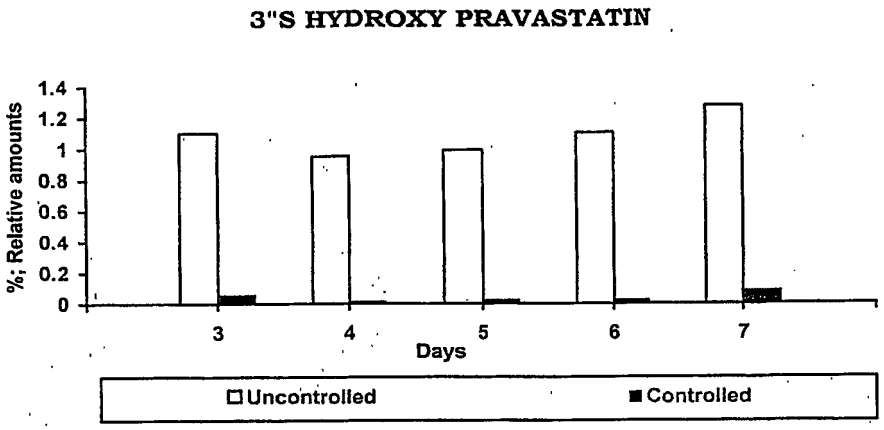


Fig 1

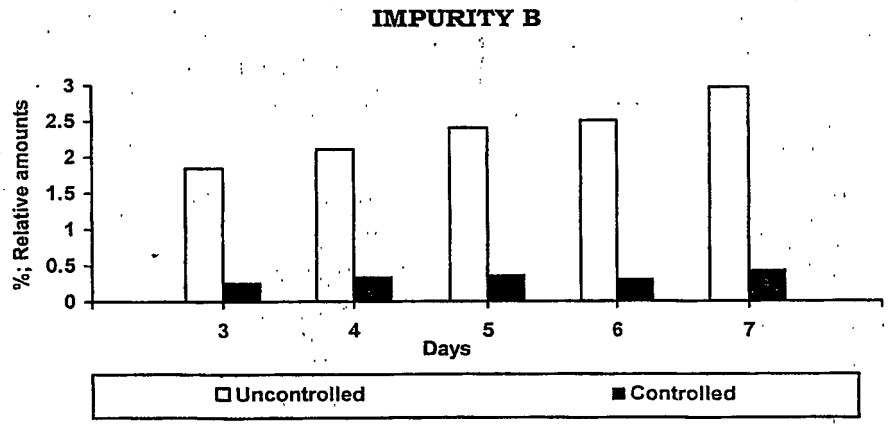


Fig 2

The results indicate that pravastatin is obtained with very low quantities of related compounds i.e. Impurity B (Formula-III) and 3"S hydroxy pravastatin (Formula-IV), when residual compactin levels were maintained between 300 and 900 µg/mL.

Experiments conducted under similar conditions except for maintaining high residual compactin levels, gave much higher quantities of related compounds. Thus, maintaining high residual levels of compactin led to lower quantities of structurally related compounds being formed. The percentage of bioconversion of compactin to pravastatin remained unaffected.

WE CLAIM:

1. A method of producing substantially pure pravastatin comprising culturing microorganism under conditions capable of converting compactin to pravastatin by maintaining residual level of compactin.
2. The process of claim 1 wherein the fermentation technique used is repeated fed-batch culture technique.
3. The process of claim 2 further comprises periodically adding quantities of compactin in the culture broth during the fermentation to maintain a residual level of compactin.
4. The process of claim 3 wherein the residual level of comapactin is maintained in the fermentation stage within the range of about 100-1800 μg/mL.
5. The process of claim 3 wherein residual level of compactin is maintained within the range of about 300-900 μg/mL.
6. The process of claim 3 wherein maintaining the residual level of compactin at 300-900 μg/mL results in about ~ 14 fold decrease in the impurity of Formula-III and ~ 7 fold decrease in impurity of Formula-IV in fermentation broth.
7. A process of claim 3 wherein the said compactin can be any soluble salt of compactin.
8. The process of claim 7 wherein compactin used is preferably sodium salt of compactin.
9. The process of claim 8 wherein the said compactin is provided in the form of solution comprising sodium salt of compactin.

10. A process of claim 3 wherein the said compactin can be semi purified or intermediate stage of compactin.

11. The process of claim 1 wherein the said microorganism belongs to *Streptomyces* species.

12. The process of claim 12 wherein the said microorganism is *Streptomyces carbophilus*.

13. The process of claim 12 wherein said microorganism is a *Streptomyces carbophilus* strain, variant or mutant thereof.

14. The process of claim 1 wherein the conditions capable of converting compactin to pravastatin comprises of fermentation production medium containing (g/L) glucose 15-23, soya bean meal 25-38, cottonseed meal 2-4, corn steep liquor 5-8, sodium chloride 5-6 and calcium carbonate 2-3.

15. The process of claim 14 wherein the temperature of the production medium is from about 18°C to 50°C.

16. The process of claim 15 wherein the temperature is from about 25°C to 30°C.

17. The process of claim 16 wherein the temperature range is from about 26°C to 28°C.

18. The process of claim 14 wherein the pH maintained is from about 5 to 10.

19. The process of claim 18 wherein the pH maintained is about 6.0 to 8.5

20. The process of claim 19 wherein the pH maintained is 7.3 to 8.0

21. The process of claim 14 wherein the shaking condition is from about 100 to 600 rpm.

22. The process of claim 21 wherein the shaking condition is from about 100 to 350 rpm.

23. The process of claim 1 wherein the percentage of conversion is from about 50 to 75%.

24. The process of claim 23 wherein the percentage of conversion is from about 65 to 75%.

25. The process of claim 24 wherein the percentage of conversion is atleast about 70%.

26. The method for producing pravastatin as herein described and exemplified by the examples.

Dated this 1ST day of **April, 2003.**

For RANBAXY LABORATORIES LIMITED

(Sushil Kumar Patawari)
Company Secretary

Ranbaxy Laboratories Limited

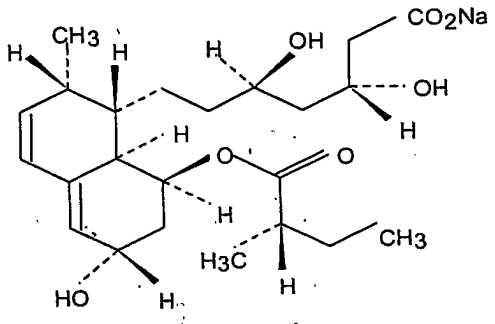
Application No.

No. of sheets = 04

Sheet 01 of 04

0569-03

- 1 APR 2003



FORMULA I

For RANBAXY LABORATORIES LIMITED

(Sushil Kumar Patawari)
Company Secretary

Ranbaxy Laboratories Limited

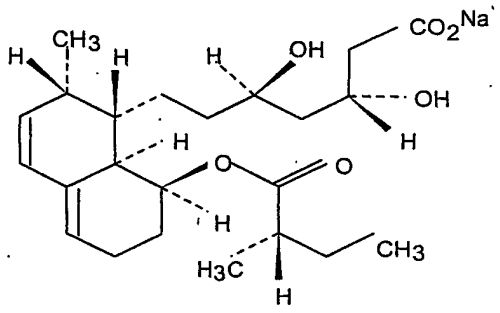
Application No.

No. of sheets = 04

Sheet 02 of 04

0559-DEL-03

- 1 APR 2003



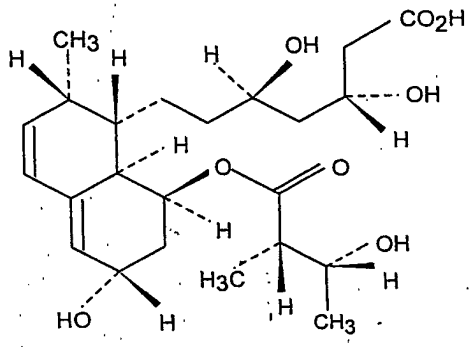
FORMULA II

For RANBAXY LABORATORIES LIMITED

(Sushil Kumar Patawari)
Company Secretary

0559-DEL-03

APR 2003



FORMULA III

For RANBAXY LABORATORIES LIMITED

(Sushil Kumar Patawari)
Company Secretary

Ranbaxy Laboratories Limited

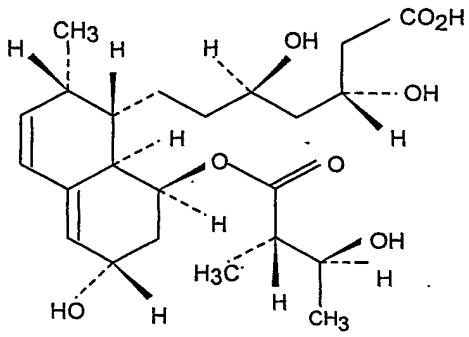
Application No.

No. of sheets = 04

Sheet 04 of 04

0559-DEL-03

APR 2003



FORMULA IV

For RANBAXY LABORATORIES LIMITED

(Sushil Kumar Patawari)
Company Secretary

FOR INAI

ABSTRACT

The present invention provides a method for producing pravastatin from compactin by microbial hydroxylation and substantially reducing the quantities of the structurally related analogues, namely 3" hydroxy pravastatin formed during the process, by maintaining a residual level of compactin.